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Article

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DNA mismatch repair-deficient (MMR-d) cancers present an abundance of neoantigens that is thought to explain their exceptional responsiveness to immune checkpoint blockade (ICB)[1,2]. Here, in contrast to other cancer types[3–5], we observed that 20 out of 21 (95%) MMR-d cancers with genomic inactivation of β2-microglobulin (encoded by *B2M*) retained responsiveness to ICB, suggesting the involvement of immune effector cells other than $CD8^+$ T cells in this context. We next identified a strong association between *B2M* inactivation and increased infiltration by γδ T cells in MMR-d cancers. These γδ T cells mainly comprised the Vδ1 and Vδ3 subsets, and expressed high levels of PD-1, other activation markers, including cytotoxic molecules, and a broad repertoire of killer-cell immunoglobulin-like receptors. In vitro, PD-1^+ γδ T cells that were isolated from MMR-d colon cancers exhibited enhanced reactivity to human leukocyte antigen (HLA)-class-I-negative MMR-d colon cancer cell lines and *B2M*-knockout patient-derived tumour organoids compared with antigen-presentation-proficient cells. By comparing paired tumour samples from patients with MMR-d colon cancer that were obtained before and after dual PD-1 and CTLA-4 blockade, we found that immune checkpoint blockade substantially increased the frequency of γδ T cells in B2M-deficient cancers. Taken together, these data indicate that γδ T cells contribute to the response to immune checkpoint blockade in patients with HLA-class-I-negative MMR-d colon cancers, and underline the potential of γδ T cells in cancer immunotherapy.

ICB targeting the PD-1–PD-L1 and/or CTLA-4 axes provides durable clinical benefits to patients who have cancers with MMR-d and high microsatellite instability[6–9]. The exceptional responses of cancers with MMR-d and high microsatellite instability to ICB is thought to be explained by their substantial burden of putative neoantigens, which originate from the extensive accumulation of mutations in their genomes[1,2]. This is consistent with the current view that PD-1 blockade mainly boosts endogenous antitumour immunity driven by $CD8^+$ T cells, which recognize HLA-class-I-bound neoepitopes on cancer cells[10–12]. However, MMR-d colon cancers frequently lose HLA-class-I-mediated antigen presentation due to silencing of HLA class I genes, inactivating mutations in β2-microglobulin (encoded by *B2M*) or other defects in the antigen processing machinery[13–16], which can render these tumours resistant to $CD8^+$ T-cell-mediated immunity[3–5,17]. Notably, early evidence has indicated that B2M-deficient, MMR-d cancers can obtain durable responses to PD-1 blockade[18], suggesting that immune cell subsets other than $CD8^+$ T cells contribute to these responses.

HLA-class-I-unrestricted immune cell subsets, which have the ability to kill tumour cells, include natural killer (NK) cells and γδ T cells. γδ T cells share many characteristics with their αβ T cell counterpart, such as cytotoxic effector functions, but express a distinct TCR that is composed of a γ and a δ chain. Different subsets of γδ T cells are defined by their TCR δ chain use, of which those expressing Vδ1 and Vδ3 are primarily 'tissue-resident' at mucosal sites, whereas those expressing Vδ2 are mainly found in blood[19]. Both adaptive and innate mechanisms of activation—for example, through stimulation of their γδ TCR or innate receptors such as NKG2D, DNAM-1, NKp30 or NKp44—have been described for γδ T cells[20]. Killer-cell immunoglobulin-like receptors (KIRs) are expressed by γδ T cells and regulate their activity depending on HLA class I expression in target cells[21]. Furthermore, γδ T cells

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were found to express high levels of PD-1 in MMR-d colorectal cancers (CRCs)[22], suggesting that these cells may be targeted by PD-1 blockade.

Here, we applied a combination of transcriptomic and imaging approaches for an in-depth analysis of ICB-naive and ICB-treated MMR-d colon cancers, as well as in vitro functional assays, and found evidence indicating that γδ T cells mediate responses to HLA-class-I-negative MMR-d tumours during treatment with ICB.

ICB is effective in *B2M*^{MUT} MMR-d cancers

We evaluated responses to PD-1 blockade therapy in a cohort of 71 patients with MMR-d cancers from various anatomical sites treated in the Drug Rediscovery Protocol (DRUP)[23] in relation to their *B2M* status (Fig. 1a, Extended Data Fig. 1a–c and Supplementary Table 1). A clinical benefit (CB; defined as at least 4 months of disease control; the primary outcome of the DRUP) was observed in 20 out of 21 (95%) of patients with tumours with mutant or deleted *B2M* (*B2M*^{MUT}) tumours versus 31 out of 50 (62%) of patients with tumours with wild-type *B2M* (*B2M*^{WT}) (two-sided Fisher's exact test, $P = 0.0038$; logistic regression, $P = 0.022$ and $P = 0.027$, adjusted for tumour mutational burden (TMB), and TMB plus tumour type, respectively; Fig. 1b). Among patients with *B2M*^{MUT} tumours, 3 out of 21 (14%) individuals experienced a complete response (according to RECIST1.1 criteria), 12 (57%) experienced a partial response, 5 (24%) experienced a durable stable disease and 1 (4.8%) experienced progressive disease as the best overall response. All 44 *B2M* alterations across 21 patients were clonal (Methods), consistent with previous observations in MMR-d cancers[18]. A total of 13 out of 21 (62%) patients with *B2M*^{MUT} tumours had biallelic *B2M* alterations, 4 (19%) had potentially biallelic alterations and 4 (19%) had non-biallelic alterations (Fig. 1c and Methods). Non-biallelic alterations have also been associated with complete loss of B2M protein expression in MMR-d tumours[18]. Thus, *B2M* alterations are associated with a high clinical benefit rate of PD-1 blockade in patients with MMR-d cancers.

Vδ1 and Vδ3 TCRs are overexpressed in *B2M*^{MUT} cancers

To gain insights into the immune cell subsets that are involved in immune responses to HLA-class-I-negative MMR-d cancers, we used data of The Cancer Genome Atlas (TCGA) and studied the transcriptomic changes associated with the genomic loss of *B2M* in three cohorts of individuals with MMR-d cancer in colon adenocarcinoma (COAD; $n = 50$ (*B2M*^{WT}), $n = 7$ (*B2M*^{MUT})), stomach adenocarcinoma (STAD; $n = 48$ (*B2M*^{WT}) and $n = 12$ (*B2M*^{MUT})), and endometrium carcinoma (UCEC; $n = 118$ (*B2M*^{WT}) and $n = 4$ (*B2M*^{MUT})). We found that *B2M* was among the most significantly downregulated genes in *B2M*^{MUT} cancers (two-sided limma-voom-based regression, $P = 3.5 \times 10^{-4}$, Benjamini–Hochberg false-discovery rate (FDR)-adjusted $P = 0.12$, adjusted for tumour type; Fig. 1d). Genes encoding components of the HLA class I antigen presentation machinery other than *B2M* were highly upregulated in *B2M*^{MUT} tumours, which may reflect reduced evolutionary pressure on somatic inactivation of these genes in the *B2M*^{MUT} context[18] (Fig. 1d). Notably, we found *TRDV1* and *TRDV3*, which encode the variable regions of the δ1 and δ3 chains of the γδ T cell receptor (TCR), among the most significantly upregulated loci in *B2M*^{MUT} tumours (*TRDV1*, two-sided limma-voom-based regression, FDR-adjusted $P = 0.00090$, adjusted for tumour type; *TRDV3*, two-sided limma-voom-based regression, FDR-adjusted $P = 0.0015$, adjusted for tumour type; Fig. 1d), regardless of the allelic status of the *B2M* alteration (Extended Data Fig. 1d). Consistent with this, the expression levels of *TRDV1* and *TRDV3* were higher in *B2M*^{MUT} compared with in *B2M*^{WT} MMR-d cancers (two-sided Wilcoxon rank-sum test, $P = 6.5 \times 10^{-8}$ for all of the cohorts combined; two-sided linear regression, $P = 4.7 \times 10^{-6}$, adjusted for tumour type; Fig. 1d–f). Moreover, *B2M*^{MUT} tumours showed overexpression of multiple KIRs (Fig. 1d), which clustered together with *TRDV1* and *TRDV3* on the basis of hierarchical clustering (Extended Data Fig. 1e). The expression level of different KIRs (Supplementary Table 2) was higher in *B2M*^{MUT} tumours compared with in *B2M*^{WT} MMR-d tumours (two-sided Wilcoxon rank-sum test, $P = 4.4 \times 10^{-6}$ for all cohorts combined; two-sided linear regression, $P = 4.7 \times 10^{-5}$, adjusted for tumour type; Fig. 1d–f). Together, these results suggest that ICB-naive *B2M*^{MUT} MMR-d cancers show increased levels of Vδ1 and Vδ3 T cells as well as increased numbers of these or other immune cells expressing KIRs—a potential mechanism of recognition of HLA class I loss.

We used marker gene sets (modified from ref. [24]; Methods and Supplementary Table 2) to estimate the abundance of a broad set of other immune cell types on the basis of the RNA expression data of the TCGA cohorts. Hierarchical clustering identified a high- and a low-infiltrated cluster in each of the three tumour types (Fig. 1e). Compared with the Vδ1 and Vδ3 T cell and KIR gene sets, the other marker gene sets showed no or only weak association between expression level and *B2M* status, indicating that our findings were not solely driven by a generally more inflamed state of *B2M*^{MUT} tumours (Fig. 1e,f and Extended Data Fig. 1f).

We next revisited the DRUP cohort and specifically applied the marker gene sets to RNA expression data. Despite the low patient numbers and high heterogeneity regarding tumour types and biopsy locations of this cohort, we confirmed increased *TRDV1* and *TRDV3* expression in *B2M*^{MUT} tumours pan-cancer (two-sided linear regression, $P = 0.017$, adjusted for tumour type and biopsy site; Fig. 1g, Extended Data Fig. 1g and Methods). KIR expression was significantly associated with *B2M* status only in CRC (Fig. 1g). Across mismatch repair-proficient (MMR-p) metastatic cancers in the Hartwig database[25], 36 out of 2,256 (1.6%) cancers had a clonal *B2M* alteration, which was frequently accompanied by loss of heterozygosity (LOH) (Extended Data Fig. 1h and Supplementary Table 3). Although rare, *B2M* alterations were also significantly associated with increased expression of *TRDV1*/*TRDV3* loci in this context (two-sided linear regression, $P = 2.2 \times 10^{-17}$, adjusted for tumour type; Extended Data Fig. 1i and Methods). Taken together, *B2M* defects are positively associated with clinical benefits of ICB treatment, as well as infiltration by Vδ1 and Vδ3 T cells and expression of KIRs.

Vδ1 and Vδ3 T cells are activated in MMR-d CRC

To investigate which γδ T cell subsets are present in MMR-d colon cancers and to determine their functional characteristics, we performed single-cell RNA-sequencing (scRNA-seq) analysis of γδ T cells isolated from five MMR-d colon cancers (Extended Data Figs. 2 and 3 and Supplementary Table 4). Three distinct Vδ subsets were identified (Fig. 2a)—Vδ1 T cells were the most prevalent (43% of γδ T cells), followed by Vδ2 (19%) and Vδ3 T cells (11%) (Fig. 2b). *PDCD1* (encoding PD-1) was predominantly expressed by Vδ1 and Vδ3 T cells, whereas Vδ1 cells expressed high levels of genes that encode activation markers such as CD39 (*ENTPD1*) and CD38 (Fig. 2c and Extended Data Fig. 2b). Furthermore, proliferating γδ T cells (expressing *MKI67*) were especially observed in the Vδ1 and Vδ3 subsets (Fig. 2c). Other distinguishing features of the Vδ1 and Vδ3 T cell subsets included the expression of genes encoding activating receptors NKp46 (encoded by *NCR1*), NKG2C (encoded by *KLRC2*) and NKG2D (encoded by *KLRK1*) (Fig. 2c). Notably, the expression of several KIRs was also higher in the Vδ1 and Vδ3 subsets as compared to Vδ2 T cells (Fig. 2c). Almost all γδ T cells displayed expression of the genes encoding granzyme B (*GZMB*), perforin (*PRF1*) and granulysin (*GNLY*) (Fig. 2c). Together, these data support a role for γδ T cells in mediating natural cytotoxic antitumour responses in HLA-class-I-negative MMR-d colon cancers.

Next, we applied imaging mass cytometry (IMC) analysis to a cohort of 17 individuals with ICB-naive MMR-d colon cancers (Supplementary Table 4). High levels of γδ T cell infiltration were observed in cancers with B2M defects as compared to B2M-proficient cancers, albeit this

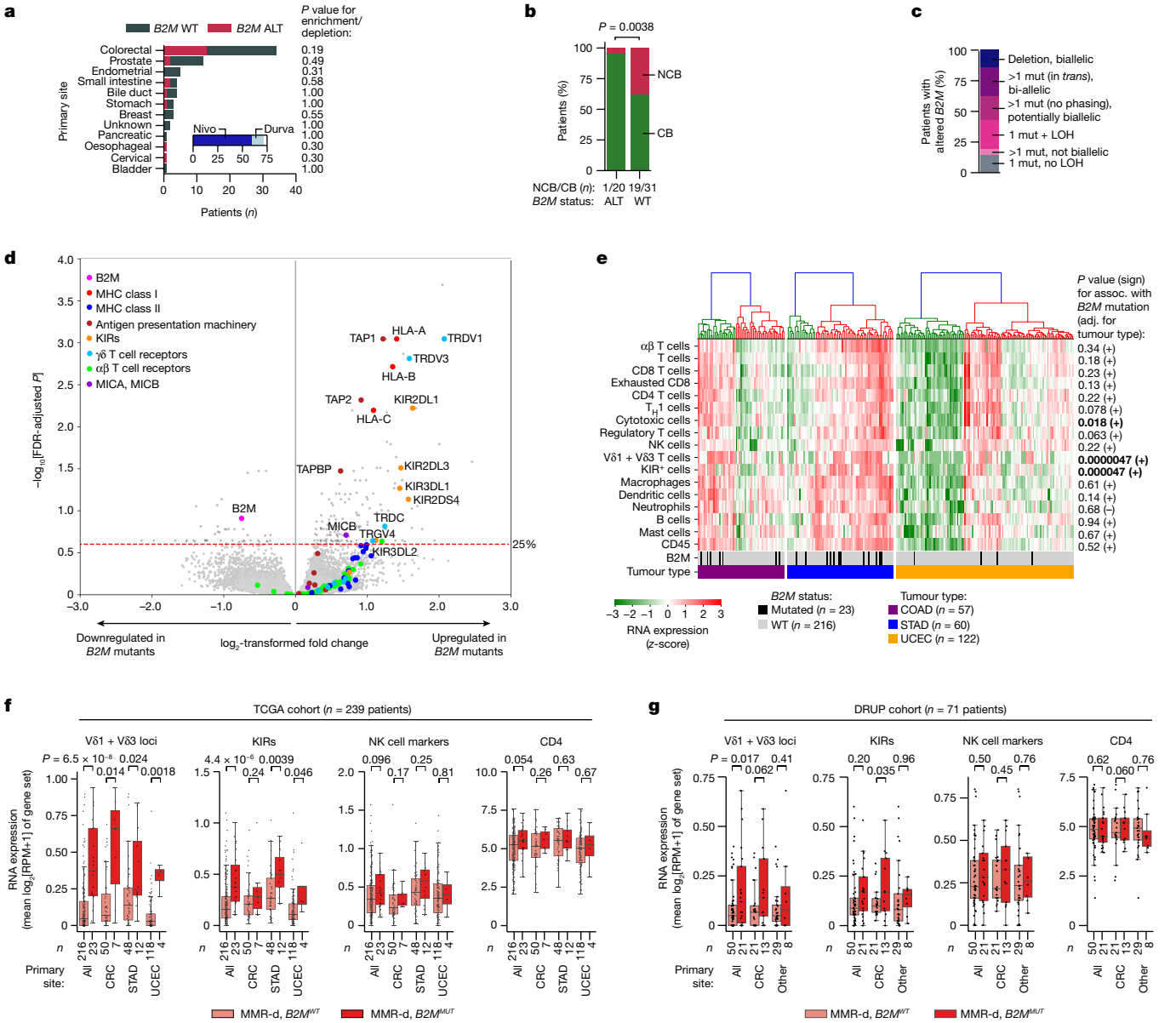

Fig. 1 | In MMR-d cancers, *B2M* defects are positively associated with ICB responsiveness and infiltration by Vδ1 and Vδ3 T cells and KIR-expressing cells. a, Tumour type distribution in the DRUP cohort ($n = 71$ patients). The colours denote patients' *B2M* status; grey, WT; red, altered (ALT). *P* values for the enrichment/depletion of *B2M*-altered tumours per primary site were calculated using two-sided Fisher's exact tests. The inset denotes the ICB treatment; dark blue, nivolumab (Nivo); light blue, durvalumab (Durva). **b**, *B2M* status (*x* axis) versus clinical benefit (green, CB; red, no clinical benefit (NCB)) of ICB treatment in the DRUP cohort. The *P* value was calculated using a two-sided Fisher's exact test. **c**, The allelic status of *B2M* alterations in the DRUP cohort. Mut, mutation. **d**, Differential gene expression between $B2M^{MUT}$ and $B2M^{WT}$ MMR-d cancers in the TCGA COAD (colon adenocarcinoma; $n = 57$ patients), STAD (stomach adenocarcinoma; $n = 60$ patients) and UCEC (uterus corpus endometrial carcinoma; $n = 122$ patients) cohorts. The results were adjusted (adj.) for tumour type and multiple-hypothesis testing (Methods). **e**, Immune marker gene set expression in MMR-d cancers of the COAD, STAD and UCEC cohorts of the TCGA. The bottom two bars indicate *B2M* status and cancer type. The association (assoc.) between gene set expression and *B2M* status was tested using ordinary least squares linear regression (adjusted for tumour type; Methods), of which two-sided *P* values and the association sign are shown on the right. Cancers were ranked on the basis of hierarchical clustering (top dendrograms). *P* values less than 0.05 are in bold. **f**, Immune marker gene set expression in $B2M^{WT}$ (pink) and $B2M^{MUT}$ (red) MMR-d cancers in the TCGA COAD, STAD and UCEC cohorts separately or combined (all). Boxes, whiskers and dots indicate the quartiles, 1.5× the interquartile range (IQR) and individual data points, respectively. *P* values were calculated using two-sided Wilcoxon rank-sum tests. **g**, Immune marker gene set expression in $B2M^{WT}$ (pink) and $B2M^{MUT}$ (red) as described in **f**, but for MMR-d cancers in the DRUP cohort. Results are shown for all cancers combined, only CRC or all non-CRC cancers (other). Two-sided *P* values were calculated using linear regression, adjusting for biopsy site and tumour type (Methods).

difference was not significant (Fig. 2d). The levels of other immune cells, including NK cells, $CD4^+$ T cells and $CD8^+$ T cells, were similar between B2M-deficient and B2M-proficient tumours (Fig. 2d). In B2M-deficient cancers, γδ T cells showed frequent intraepithelial localization and expression of CD103 (tissue-residency), CD39 (activation), granzyme B (cytotoxicity) and Ki-67 (proliferation), as well as PD-1 (Fig. 2d–f and Extended Data Fig. 2c), consistent with the scRNA-seq data. Notably, γδ T cells in B2M-deficient cancers showed co-expression of CD103 and CD39 (Extended Data Fig. 2d), which has been reported to identify tumour-reactive $CD8^+$ αβ T cells in a variety of cancers[26].

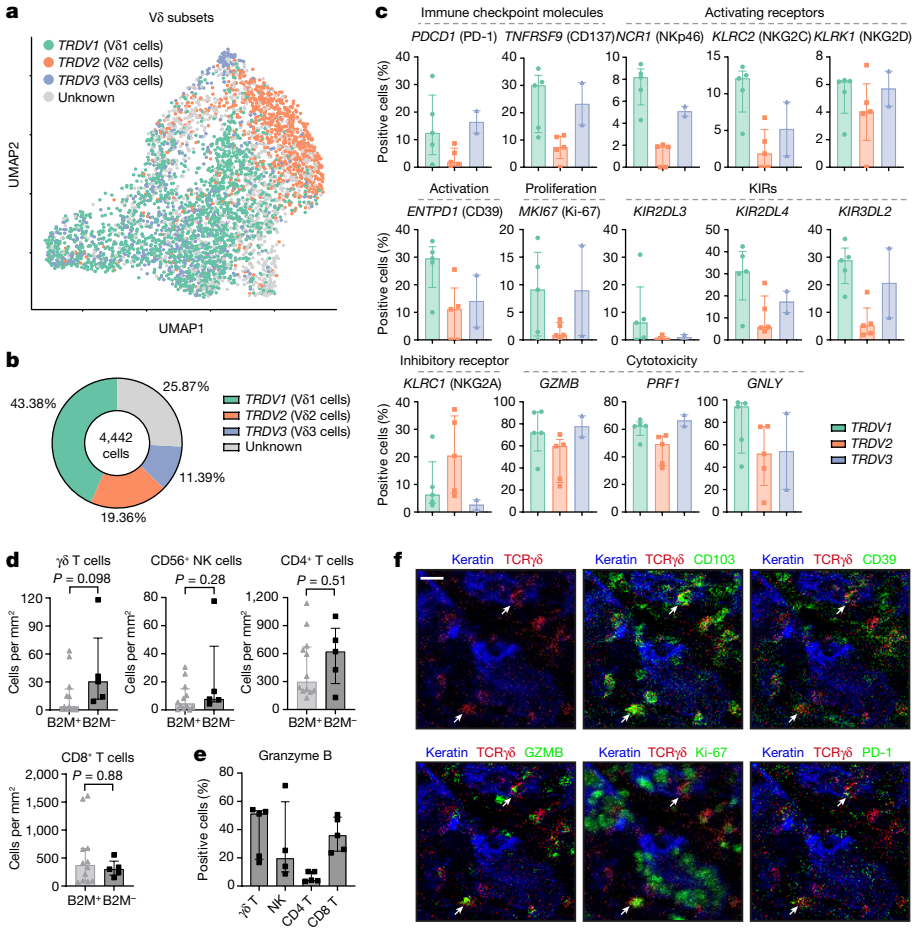

Fig. 2 | Tumour-infiltrating Vδ1 and Vδ3 T cell subsets display hallmarks of cytotoxic activity in MMR-d colon cancers. a, UMAP embedding showing the clustering of γδ T cells ($n = 4,442$) isolated from MMR-d colon cancers ($n = 5$) analysed using scRNA-seq. The colours represent the TCR Vδ chain usage. The functionally distinct γδ T cell clusters are shown in Extended Data Fig. 3. Dots represent single cells. **b**, The frequencies of TCR Vδ chain use of the γδ T cells ($n = 4,442$) analysed using scRNA-seq as a percentage of total γδ T cells. **c**, The frequencies of positive cells for selected genes across Vδ1 ($n = 1,927$), Vδ2 ($n = 860$) and Vδ3 ($n = 506$) cells as the percentage of total γδ T cells from each MMR-d colon tumour ($n = 5$) analysed using scRNA-seq. Vδ3 cells were present in two out of five colon cancers. Data are median ± IQR, with individual samples (dots). **d**, The frequencies of γδ T cells, CD56$^+$ NK cells, CD4$^+$ T cells and CD8$^+$ T cells in treatment-naive B2M$^+$ ($n = 12$) and B2M$^-$ ($n = 5$) MMR-d colon cancers. Data are median ± IQR, with individual samples (dots). *P* values were calculated using two-sided Wilcoxon rank-sum tests. **e**, The frequencies of granzyme-B-positive γδ T cells, CD56$^+$ NK cells, CD4$^+$ T cells and CD8$^+$ T cells in treatment-naive B2M$^-$ ($n = 5$) MMR-d colon cancers. CD56$^+$ NK cells were present in four out of five B2M$^-$ cancer samples. Data are median ± IQR, with individual samples (dots). **f**, Representative images of the detection of tissue-resident (CD103$^+$), activated (CD39$^+$), cytotoxic (granzyme B$^+$), proliferating (Ki-67$^+$) and PD-1$^+$ γδ T cells (white arrows) by IMC analysis of a treatment-naive MMR-d colon cancer with B2M defects. Scale bar, 20 µm.

PD-1$^+$ Vδ1 and Vδ3 T cells kill HLA-class-I$^-$ CRC cells

We next sought to determine whether tumour-infiltrating γδ T cells can recognize and kill CRC cells. We isolated and expanded PD-1$^-$ and PD-1$^+$ γδ T cells from five MMR-d colon cancers (Extended Data Fig. 4a–c and Supplementary Table 4). Consistent with the scRNA-seq data, expanded PD-1$^+$ γδ T cell populations lacked Vδ2$^+$ cells and comprised the Vδ1$^+$ or Vδ3$^+$ subsets, whereas the PD-1$^-$ fractions contained Vδ2$^+$ or a mixture of Vδ1$^+$, Vδ2$^+$ and Vδ3$^+$ populations (Fig. 3a and Extended Data Fig. 4d). Detailed immunophenotyping of the expanded γδ T cells (Fig. 3a and Extended Data Fig. 5a) showed that all of the subsets expressed the activating receptor NKG2D, whereas the surface expression of natural cytotoxicity receptors (NCRs) and KIRs was most frequent on PD-1$^+$ γδ T cells (Vδ1 or Vδ3$^+$), consistent with the scRNA-seq results of unexpanded populations.

We measured the reactivity of the expanded γδ T cell populations to HLA-class-I-negative and HLA-class-I-positive cancer cell lines (Fig. 3b and Extended Data Fig. 4b). After co-culture with the different cancer cell lines, reactivity (assessed by expression of activation markers and secretion of IFNγ) was largely restricted to PD-1$^+$ γδ T cells (Vδ1 or Vδ3$^+$), whereas activation of PD-1$^-$ γδ T cells (Vδ2$^+$) was generally not detected (Fig. 3c and Extended Data Fig. 4). PD-1$^+$ γδ T cell (Vδ1 or Vδ3$^+$) reactivity was variable and was observed against both HLA-class-I-negative and HLA-class-I-positive cell lines (Fig. 3c and Extended Data Fig. 4). To quantify and visualize the differences in the killing of CRC cell lines by PD-1$^+$ and PD-1$^-$ γδ T cells, we co-cultured the γδ T cell populations with three CRC cell lines (HCT-15, LoVo, HT-29) in the presence of a fluorescent cleaved-caspase-3/7 reporter to measure cancer cell apoptosis over time (Fig. 3d,e). We found pronounced cancer cell apoptosis after co-culture with PD-1$^+$ γδ T cells (Vδ1 or Vδ3$^+$) compared with PD-1$^-$ cells; cancer cell death was more pronounced in HLA-class-I-negative HCT-15 cells (Fig. 3e and Supplementary Videos 1 and 2). Reintroduction of *B2M* in the *B2M*-deficient HCT-15 and LoVo cells diminished their killing by PD-1$^+$ γδ T cells (Vδ1 or Vδ3$^+$) cells (Extended Data Fig. 6), suggesting that *B2M* loss increases the sensitivity to γδ T cells.

Next, we established two parental patient-derived tumour organoid lines (PDTOs; Supplementary Table 5) of MMR-d CRC and generated isogenic *B2M*^{KO} lines using CRISPR. Genomic knockout of *B2M*

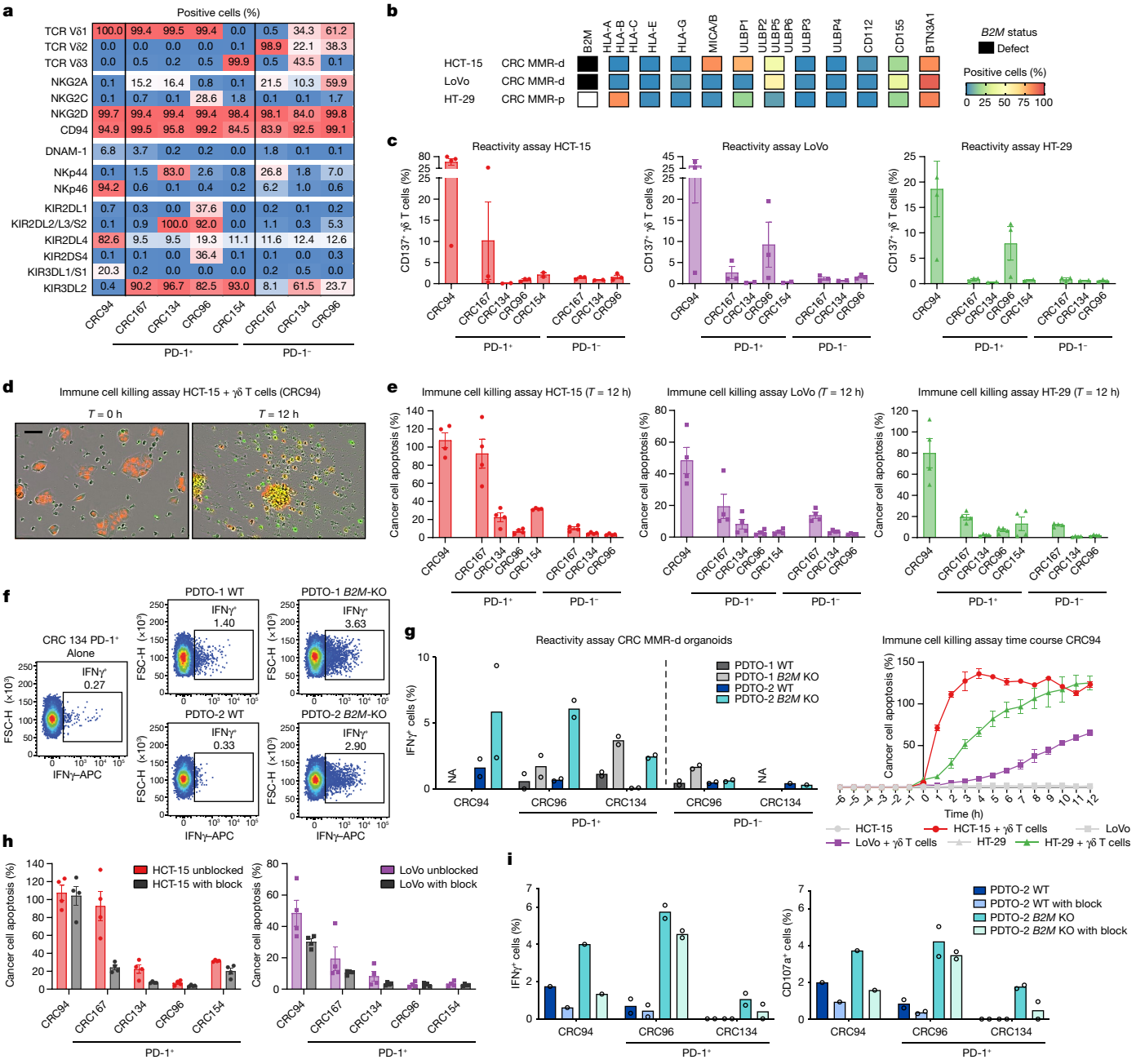

Fig. 3 | γδ T cells from MMR-d colon cancers show preferential reactivity to HLA-class-I-negative cancer cell lines and organoids. a, The percentage of positive cells for the indicated markers on expanded γδ T cells from MMR-d colon cancers ($n = 5$). **b**, Diagram showing the *B2M* status and surface expression of HLA class I, NKG2D ligands, DNAM-1 ligands and butyrophilin on CRC cell lines. MMR-p, MMR proficient. **c**, CD137 expression on γδ T cells after co-culture with CRC cell lines. Data are mean ± s.e.m. from at least two independent experiments. **d**, Representative images showing the killing of NucLightRed-transduced HCT-15 cells by γδ T cells in the presence of a green fluorescent caspase-3/7 reagent. Cancer cell apoptosis is visualized in yellow. Scale bar, 50 µm. **e**, Quantification of the killing of CRC cell lines after co-culture with γδ T cells as described in **d**. Data are mean ± s.e.m. of two wells with two images per well. A representative time course of cancer cell apoptosis is shown at the bottom right. **f**, Representative flow cytometry plots showing IFNγ expression in γδ T cells unstimulated (alone) and after stimulation with two $B2M^{WT}$ and $B2M^{KO}$ CRC MMR-d organoids. **g**, IFNγ expression in γδ T cells after stimulation with two $B2M^{WT}$ and $B2M^{KO}$ CRC MMR-d organoids, shown as the difference compared with the unstimulated γδ T cell sample. Data are from two biological replicates, except for a single biological replicate of CRC134 PD-1$^-$. NA, not available. **h**, The killing of CRC cell lines after 12 h co-culture with γδ T cells with or without NKG2D ligand blocking. Data are mean ± s.e.m. of two wells with two images per well. **i**, IFNγ (left) and CD107a (right) expression in γδ T cells after stimulation with $B2M^{WT}$ PDTO-2 or $B2M^{KO}$ PDTO-2, with or without NKG2D ligand blocking and subtracted background signal. Data are from two biological replicates, except for a single biological replicate of CRC94.

effectively abrogated cell surface expression of HLA class I (Extended Data Fig. 7). We exposed two $B2M^{KO}$ lines and their parental $B2M^{WT}$ lines to the expanded γδ T cell subsets, and quantified γδ T cell activation by determination of IFNγ expression. Similar to our cell line data, γδ T cells displayed increased reactivity to $B2M^{KO}$ PDTOs in comparison to the $B2M^{WT}$ PDTOs (Fig. 3f,g). Furthermore, γδ T cell reactivity to $B2M^{KO}$ tumour organoids was preferentially contained within the PD-1$^+$ population of γδ T cells (Fig. 3g). Thus, a lack of HLA class I antigen presentation in MMR-d tumour cells can be effectively sensed by γδ T cells and stimulates their antitumour response.

Expression of NKG2D on γδ T cells decreased during co-culture with target cells (Extended Data Fig. 8a,b), suggesting the involvement of the NKG2D receptor in γδ T cell activity. The NKG2D ligands MICA/B and ULBPs were expressed by the cancer cell lines (Fig. 3b) and the MMR-d CRC PDTOs, irrespective of their *B2M* status (Extended Data Fig. 7). To examine which receptor–ligand interactions might regulate the activity of PD-1$^+$ γδ T cells, we performed blocking experiments focused on (1) NKG2D, (2) DNAM-1 and (3) γδ TCR signalling. Of these candidates, the only consistent inhibitory effect was observed for NKG2D ligand blocking on cancer cells, which decreased the activation and killing ability of most PD-1$^+$ γδ T cells (Fig. 3h and Extended Data Fig. 8c,d), confirming the mechanistic involvement of the NKG2D receptor in γδ T cell activation in this context. Moreover, blocking NKG2D ligands on MMR-d CRC PDTOs reduced the PDTO-directed tumour reactivity of γδ T cells from CRC94 and CRC134 (Fig. 3i). Together, these results show that γδ T cell reactivity to MMR-d tumours is partly dependent on NKG2D/NKG2D-ligand interactions.

ICB boosts Vδ1 and Vδ3 T cells in *B2M^{MUT}* CRC

We subsequently studied how ICB influences γδ T cell infiltration and activation in MMR-d colon cancers in the therapeutic context. For this purpose, we analysed pre- and post-treatment samples of the NICHE trial[9], in which patients with colon cancer were treated with neoadjuvant PD-1 plus CTLA-4 blockade. Consistent with our observations in the DRUP cohort, 4 out of 5 (80%) individuals with *B2M^{MUT}* cancers in the NICHE trial showed a complete pathologic clinical response. Immunohistochemical analysis confirmed the loss of B2M protein expression on tumour cells in all mutated cases (Extended Data Fig. 9). Whereas expression of immune marker gene sets in the pretreatment samples was similar between 5 *B2M^{MUT}* versus 13 *B2M^{WT}* cancers, ICB induced a clear immunological divergence between these two groups (Fig. 4a). The *B2M^{MUT}* subgroup was most significantly associated with higher post-treatment expression of *TRDV1* and *TRDV3* (two-sided Wilcoxon rank-sum test, $P = 0.0067$; Fig. 4a), followed by higher expression of the general immune cell marker CD45, NK-cell-related markers, KIRs and αβTCRs (two-sided Wilcoxon rank-sum test, $P = 0.016$, $P = 0.016$, $P = 0.027$ and $P = 0.043$, respectively; Fig. 4a and Extended Data Fig. 10a). The set of KIRs upregulated after ICB in *B2M^{MUT}* cancers (Extended Data Fig. 10b) was consistent with the sets of KIRs upregulated in *B2M^{MUT}* MMR-d cancers in TCGA (Fig. 1e), and those expressed by MMR-d tumour-infiltrating γδ T cells (Fig. 2c). Pre- and post-ICB gene expression levels related to CD4 and CD8 infiltration were not associated with *B2M* status (Fig. 4a and Extended Data Fig. 10a).

To quantify and investigate the differences in immune profiles after ICB treatment, we used IMC to analyse tissues derived from five *B2M^{MUT}* HLA-class-I-negative and five *B2M^{WT}* HLA-class-I-positive cancers before and after ICB treatment. In the ICB-naive setting, *B2M^{MUT}* MMR-d colon cancers showed higher γδ T cell infiltration compared with *B2M^{WT}* MMR-d colon cancers (two-sided Wilcoxon rank-sum test, $P = 0.032$; Fig. 4b and Extended Data Fig. 10c). Importantly, a large proportion of these γδ T cells showed an intraepithelial localization in *B2M^{MUT}* MMR-d colon cancers compared with the *B2M^{WT}* samples (two-sided Wilcoxon rank-sum test, $P = 0.0079$; Extended Data Fig. 10d). No significant differences were observed in the infiltration of other immune cells, such as NK cells, CD4$^+$ T cells and CD8$^+$ T cells, in ICB-naive *B2M^{MUT}* versus *B2M^{WT}* MMR-d colon cancers (Fig. 4b). ICB treatment resulted in major pathologic clinical responses, and residual cancer cells were absent in most post-ICB samples. All post-ICB tissues showed a profound infiltration of different types of immune cells (Extended Data Fig. 10e), of which γδ T cells were the only immune subset that was significantly higher in ICB-treated *B2M^{MUT}* compared with *B2M^{WT}* MMR-d colon cancers (two-sided Wilcoxon rank-sum test, $P = 0.016$; Fig. 4b and Extended Data Fig. 10c). In the sole *B2M^{MUT}* case that still contained cancer cells after treatment with ICB, the majority of granzyme B$^+$ immune cells infiltrating the tumour epithelium were γδ T cells (Fig. 4c). These γδ T cells displayed co-expression of CD103, CD39, Ki-67 and PD-1 (Extended Data Fig. 10f–h). Taken together, these results show that ICB treatment of MMR-d colon cancer increases the presence of activated, cytotoxic and proliferating γδ T cells at the tumour site, especially when these cancers are B2M-deficient, highlighting γδ T cells as effectors of ICB treatment within this context.

Discussion

CD8$^+$ αβ T cells are major effectors of ICB[11,12,27] and rely on HLA class I antigen presentation of target cells. We confirm and shed light on the paradox that patients with HLA class I defects in MMR-d cancers retain the clinical benefit of ICB, suggesting that other immune effector cells are involved in compensating for the lack of conventional CD8$^+$ T cell immunity in this setting. We show that genomic inactivation of *B2M* in MMR-d colon cancers was associated with: (1) an elevated frequency of activated γδ T cells in ICB-naive tumours; (2) an increased presence of tumour-infiltrating γδ T cells after ICB treatment; (3) in vitro activation of tumour-infiltrating γδ T cells by CRC cell lines and PDTOs; and (4) killing of tumour cell lines by γδ T cells, in particular by Vδ1 and Vδ3 subsets expressing PD-1.

Different subsets of γδ T cells exhibit substantially diverse functions that, in the context of cancer, range from tumour-promoting to tumouricidal effects[20,28,29]. Thus, it is of interest to determine what defines antitumour reactivity of γδ T cells. Here we isolated Vδ1/3-expressing PD-1$^+$ T cells as well as Vδ2-expressing PD-1$^-$ T cells from MMR-d tumour tissues. Our data suggest that especially tumour-infiltrating Vδ1 and Vδ3 T cells can recognize and kill HLA-class-I-negative MMR-d tumours, whereas Vγ9Vδ2 cells, the most studied and main subset of γδ T cells in the blood, appear to be less relevant within this context. This is consistent with other studies showing that the cytotoxic ability of Vδ1 cells generally outperforms their Vδ2 counterparts[30–34]. Notably, reports of the cytotoxicity of tumour-infiltrating Vδ3 cells have been lacking. Furthermore, the observation that PD-1$^+$ γδ T cells (Vδ1 and Vδ3 phenotype) demonstrated clearly higher levels of antitumour reactivity compared with their PD-1$^-$ counterparts (Vδ2 phenotype) suggests that, as for CD8$^+$ αβ T cells[35], PD-1 expression may be a marker of antitumour reactivity in γδ T cells.

The mechanisms of activation of γδ T cells are notoriously complex and diverse[20]. Specifically, for Vδ1$^+$ cells, NKG2D has been described to be involved in tumour recognition, which is dependent on tumour cell expression of NKG2D ligands MICA/B and ULBPs[36–38]. Here, MICA/B and ULBPs were highly expressed by the MMR-d CRC cell lines and tumour organoids, and blocking these ligands reduced γδ T cell activation and cytotoxicity. This suggests a role for the activating receptor NKG2D in γδ T cell immunity to MMR-d tumours. Future research should address the outstanding question of how γδ T cells accumulate in B2M-deficient tumours, and whether the lack of CD8$^+$ T cell activity might contribute to the establishment of an attractive niche for γδ T cells and other immune effector cells. Potential mechanisms for the recognition of HLA-class-I-negative phenotypes may include KIR-, NKG2A- and LILRB1-mediated interactions with target cancer cells. Notably, we found that the expression of KIRs was most pronounced on PD-1$^+$ γδ T cells (Vδ1 or Vδ3$^+$ subsets), which demonstrated anti-tumour activity. Whether the lack of KIR-mediated signalling promotes the survival of γδ T cells and their intratumoural proliferation remains to be studied.

Our findings have broad implications for cancer immunotherapy. First, our findings strengthen the rationale for combining PD-1 blockade with immunotherapeutic approaches to further enhance γδ T-cell-based antitumour immunity. Second, the presence or absence in tumours of specific γδ T cell subsets (such as Vδ1 or Vδ3) may help to define patients who are responsive or unresponsive to ICB, respectively, especially in the case of MMR-d cancers and

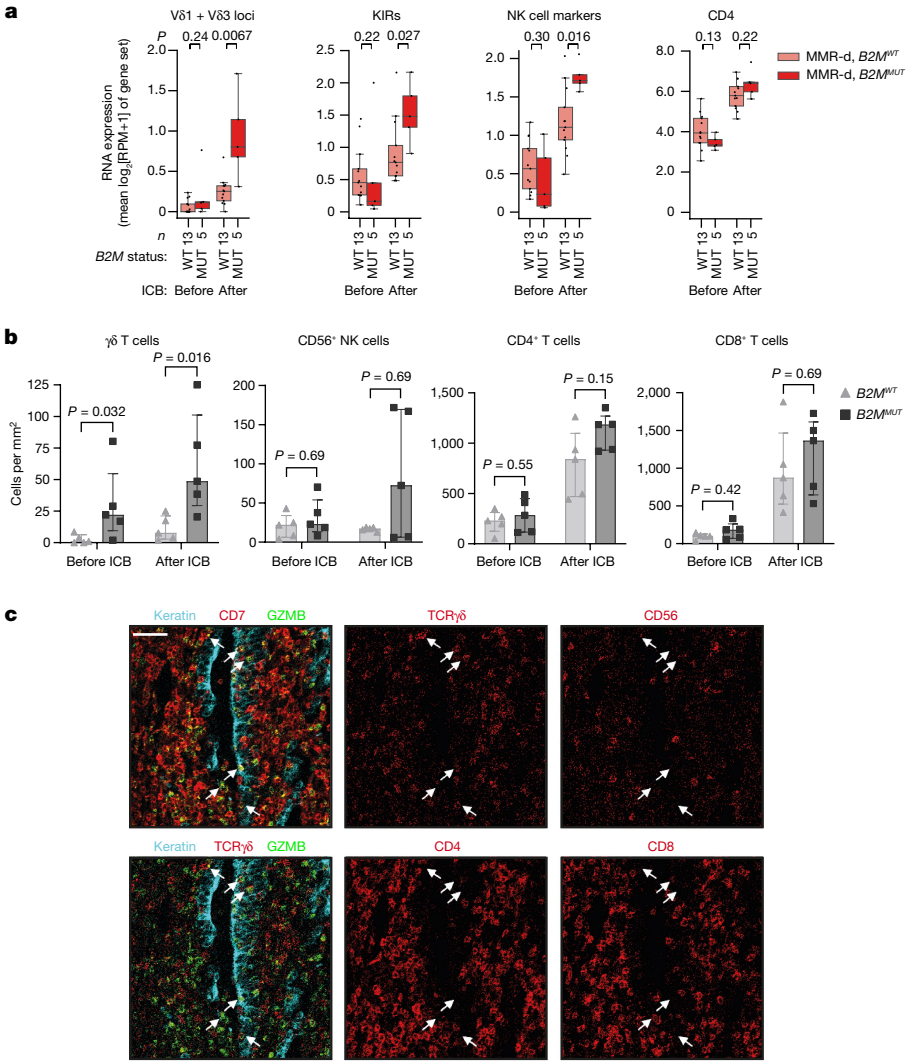

Fig. 4 | ICB induces substantial infiltration of γδ T cells into MMR-d colon cancers with defects in antigen presentation. a, The RNA expression of different immune marker gene sets in MMR-d $B2M^{WT}$ (pink) and MMR-d $B2M^{MUT}$ (red) cancers before (left) and after (right) neoadjuvant ICB in the NICHE study. The boxes, whiskers and dots indicate quartiles, 1.5 × IQR and individual data points, respectively. *P* values were calculated using two-sided Wilcoxon rank-sum tests comparing MMR-d $B2M^{WT}$ versus MMR-d $B2M^{MUT}$ cancers. **b**, The frequencies of γδ T cells, $CD56^+$ NK cells, $CD4^+$ T cells and $CD8^+$ T cells in $B2M^{WT}$ ($n = 5$) and $B2M^{MUT}$ ($n = 5$) MMR-d colon cancers before and after ICB treatment. Data are median ± IQR, with individual samples (dots). *P* values were calculated using two-sided Wilcoxon rank-sum tests. **c**, Representative images of granzyme-B-positive γδ T cells infiltrating the tumour epithelium (white arrows) by IMC analysis of a $B2M^{MUT}$ MMR-d colon cancer after ICB treatment. Scale bar, 50 µm.

other malignancies with frequent HLA class I defects, such as stomach adenocarcinoma[39] and Hodgkin's lymphoma[40]. Third, our results suggest that MMR-d cancers and other tumours with HLA class I defects may be particularly attractive targets for Vδ1 or Vδ3 T-cell-based cellular therapies.

Although we have provided detailed and multidimensional analyses, it is probable that γδ T cells are not the only factor driving ICB responses in HLA-class-I-negative MMR-d CRC tumours. In this context, other HLA-class-I-independent immune subsets, such as NK cells and neoantigen-specific $CD4^+$ T cells may also contribute. The latter were shown to have an important role in the response to ICB (as reported in mouse *B2M*-deficient MMR-d cancer models[41]), and may also support γδ T-cell-driven responses. Notably, no subset equivalent to Vδ1 or Vδ3 T cells has been identified in mice, which complicates their investigation in in vivo models. In conclusion, our results provide strong evidence that γδ T cells are cytotoxic effector cells of ICB treatment in HLA-class-I-negative MMR-d colon cancers, with implications for further exploitation of γδ T cells in cancer immunotherapy.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-022-05593-1.

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